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MODULATION BY SUBSTRATE AND CATIONS OF GUANYLATE CYCLASE ACTIVITY IN DETERGENT-DISPERSED PLASMA MEMBRANES FROM RAT ADIPOCYTES

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SUMMARY: Guanylate cyclase activity in Triton X-100-treated plasma membranes exhibits sigmoidal profiles as a function of MgGTP, irrespective of the excess Mg^{2+}(*) concentration. In contrast, at low excess Mn^{2+} (0.2 mM) the activity *vs* substrate (MnGTP) concentration profile corresponds to a michaelian behaviour. In addition the enzyme does not require similar excess Mn^{2+} and Mg^{2+} for optimal activity at various substrate concentrations. Moreover, low concentrations of Ca^{2+} are capable of stimulating guanylate cyclase activity with Mg^{2+} as the major divalent cation.

INTRODUCTION:

Guanylate cyclase (EC 4.6.1.2.) activity in most tissues and organs is localized in both soluble and particulate subcellular fractions; such is the case with the adipocyte (2). The plasma membrane of this cell exhibits guanylate cyclase activity of high specific activity, which moreover is strongly enhanced by pre-incubation with the mild non-ionic detergent Triton X-100 (3). In addition to its different possible effects on the enzyme (4), the contaminating peroxides of this detergent (5) may also, in part be responsible for guanylate cyclase activation (6-9). It is now well established that, although native guanylate cyclase activity prefers MnGTP as a substrate, MgGTP may function equally as well under various activated situations (10, for a review). We have thus, undertaken an extensive comparison of the Mn^{2+}- and Mg^{2+}-dependent guanylate cyclase activities in detergent-dispersed plasma membranes from rat adipocytes.

(*) Excess Me^{2+} refers to levels of metal cations (Me^{2+}) above equimolar GTP and $MeCl_2$ concentrations; this parameter was emphasized by Cleland (1).

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As in other systems (10), extracellular calcium is required for the expression of basal and hormone-stimulated cyclic GMP accumulation in the adipocyte (11). We have therefore also investigated guanylate cyclase activity in the presence of low concentrations of this cation in our system.

The differences in guanylate cyclase activity observed with the three cations will be discussed in relation to their possible regulatory role *in vivo*.

METHODS:

Plasma membranes from rat adipocytes (12) were isolated as previously described (13).

Guanylate cyclase activity was determined as described in an earlier report (14). Incubation was carried out at 37°C in a final volume of 50 µl consisting of: 50 mM Tris-HCl,(pH 7.8), 1 mM dithiothreitol (at this concentration no stimulatory or inhibitory effect of this compound in the presence of Mn^{2+}, Mg^{2+} or Ca^{2+} was noted), 1mM cyclic GMP, 10 mM creatine phosphate, 1 IU phosphocreatine kinase, 0.5 µCi [α-^{32}P] GTP and the concentrations of GTP and cations indicated in the legends to figures. The reaction was initiated by addition of plasma membranes (1-5 µg protein), in their native form or preincubated for 30 min. at 0°C (about 0.5 mg/ml membrane protein) in 1% Triton X-100 (w/v) (final concentration in assay: 0.2%). The reaction was stopped by addition of 0.2 ml N HCl and [^{32}P] cyclic GMP was isolated by a double column procedure (14). Values are means of triplicate determinations of representative experiments which were performed with 2 or 3 different membrane preparations.

Although significantly inhibited in the presence of Triton, membrane phosphohydrolases were still capable of some GTP hydrolysis; thus residual amount of the nucleotide at the end of the incubation period was verified under all the conditions used; for that purpose thin-layer chromatography of the reaction mixture was performed on PEI-cellulose plates with 1M LiCl as a solvent. A major proportion of the GTP which was hydrolyzed was converted into GMP and therefore could not be reversibly transformed via the regenerating system to GTP. Accordingly, the amount of protein employed was sufficiently low to ensure that more than 80% GTP was still remaining (especially when Mg^{2+} was present).

Protein concentration was determined according to Lowry (15). All cations were in the chloride form. The source of the reagents has been listed previously (14).

RESULTS:

Guanylate cyclase activity in rat adipocyte plasma membranes assayed in the presence of Mg^{2+} as sole cation amounted to some 27% of that obtained with Mn^{2+} (Table 1); this is a rather high proportion in comparison to other systems (see 10, for references). Preincubation of plasma membranes with 1% Triton X-100 resulted in an 8-fold and 23-fold stimulation of guanylate cyclase acti-

TABLE 1: Guanylate cyclase activity in native and Triton X-100-dispersed plasma membranes from rat adipocytes.

Membrane pretreatment	metal cation Mn^{2+}	Mg^{2+}	
	specific activity ($pmol.min^{-1}.mg^{-1}$)		% Mn^{2+} activity
none	93.7 ± 40.8(3)	25.0 ± 8.7(3)	26.7%
Triton X-100	787.9 ± 242.5(7)	572.0 ± 153.2(5)	72.6%

Plasma membranes, preincubated for 30 min. at 0°C in the absence (5µg protein) or in the presence of 1% Triton X-100 (1.5 µg protein), were then assayed for guanylate cyclase activity (30 min. at 37°C). Mn^{2+}-supported activity was determined with a 0.2 mM excess of $MnCl_2$ over MnGTP (2 mM) and Mg^{2+}-supported activity with a 5 mM excess of $MgCl_2$ over MgGTP (5 mM). Results are expressed as the mean of several experiments ± S.D. (number in parentheses) from different membrane preparations.

vity at the optimal concentration (see below) of $MnCl_2$ and $MgCl_2$ respectively. Maximal activities, therfore became similar. Comparison of the substrate and cation requirements of this activated enzyme was subsequently undertaken.

Figure 1 shows that the Mn^{2+}-dependency of guanylate cyclase activity was more complex than that of Mg^{2+}. Cyclase activity exhibited a bell-shaped profile as a function of excess Mg^{2+} (optimal 1-5mM), irrespective of the substrate (MgGTP) concentration. In contrast, an excess of Mn^{2+} which was 2 orders of magnitude lower than the optimal excess Mg^{2+} concentration, was sufficient to elicit maximal activity at low MnGTP levels. At high MnGTP concentrations, activity was no longer activated by excess $MnCl_2$. Both cations were inhibitory beyond their maximal effects. Such profiles have also been observed in studies of detergent-treated enzyme from sea urchin sperm using MnGTP as substrate (16).

The substrate-dependency of guanylate cyclase activity, at the optimal concentrations of 0.2 mM Mn^{2+} and 5 mM Mg^{2+} in excess of MnGTP and MgGTP respectively, is shown in figure 2. A sigmoidal profile is observed as a function of MgGTP, whereas increa-

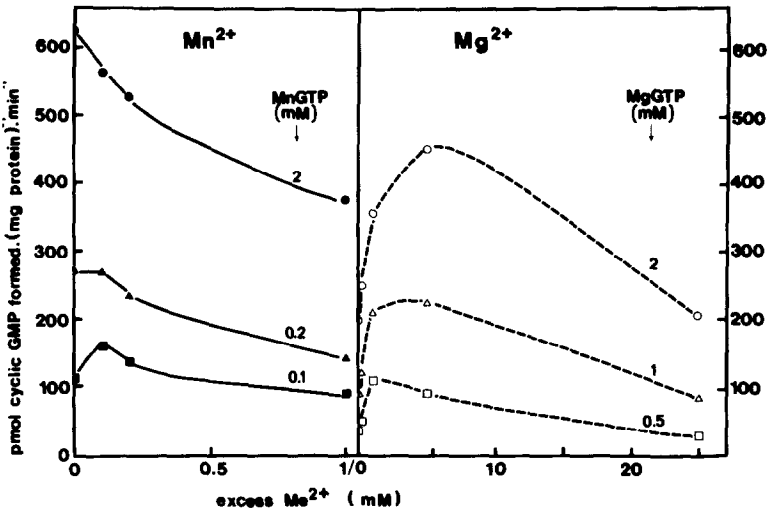

Figure 1. Effects of excess Mn^{2+} and Mg^{2+} concentrations, at different substrate concentrations, on guanylate cyclase activity in Triton-dispersed plasma membranes from rat adipocytes.

Incubations corresponding to Mn^{2+}- and Mg^{2+}-dependent activities were carried out with, respectively 1.7 µg membrane protein for 15 min. and 2.8 µg membrane protein for 30 min.

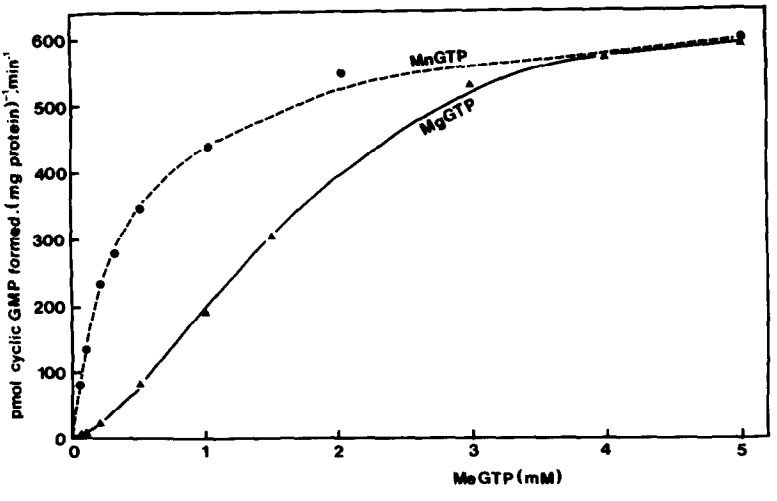

Figure 2. Substrate-dependency of guanylate cyclase activity in Triton-dispersed plasma membranes from rat adipocytes.

MnGTP (●—●) or MgGTP (▲—▲) varied respectively, in the presence of a constant excess of 0.2 mM Mn^{2+} or 5 mM Mg^{2+}, in the same conditions as in fig.1.

sing MnGTP concentration results in a classical hyperbolic profile. Identical profiles were obtained with the 100,000 *g* supernatant from Triton-solubilized plasma membranes (not shown). There was a 5-6 fold increase in substrate concentration leading to half-maximal velocity ($S_{0.5}$ value) in the case of MgGTP as compared to that obtained for MnGTP; values were 1.40 ± 0.32 mM and 0.25 ± 0.10 mM respectively. Table 2 summarizes the Hill coefficients for MnGTP and MgGTP obtained at different excess cation concentrations: only at low excess Mn^{2+} was the Hill coefficient equal to unity. $S_{0.5}$ values were not significantly affected as Me^{2+} varied.

The effects of calcium on Mg^{2+}- and Mn^{2+}-dependent guanylate cyclase activities are shown in figure 3. Use of 0.1 mM MgGTP at optimal Mg^{2+} concentration resulted in a dose-dependent activation in the range 3 µM-300 µM Ca^{2+}; no stimulation was observed at lower excess Mg^{2+} levels. Similar activation was also observed at higher MgGTP concentrations, provided the Mg^{2+} concentration was consequently increased, such that the Mg^{2+}/MgGTP ratio remained elevated. Thus, the activation could not be explained by release of free Mg^{2+} from MgGTP. As a consequence of such effects, n_H value for MgGTP decreased in the presence of Ca^{2+}. The same results were obtained with plasma membranes which were prepared in the presence of 1 mM EGTA after the collagenase step. The absence of the regenerating system was without effect on the degree of activation elicited by Ca^{2+}. In contrast, no activation by Ca^{2+} was obtained when MnGTP was the substrate, irrespective of the excess Mn^{2+} concentration, and even at suboptimal Mn^{2+}. Lanthanum ion (La^{3+}) strictly mimicked the activatory effect of Ca^{2+} at high Mg^{2+}/MgGTP ratio; this ion was also without effect at low Mg^{2+} concentration and in the presence of Mn^{2+} (data not shown). No activity was detectable with CaGTP at various excess Ca^{2+}.

DISCUSSION:

The data presented in this report show that stimulated guanylate cyclase activity in detergent-dispersed plasma membranes from rat adipocytes is highly sensitive to the concentrations and nature of substrate and divalent cations. Therefore conditions altering their availibility and/or accessibility to the membrane would lead to a fine modulation of guanylate cyclase activity.

TABLE 2: Hill coefficients for MnGTP and MgGTP as a function of excess Me^{2+}.

excess Me^{2+} (mM)	n_H value Mn^{2+}	Mg^{2+}
0	1.64 ± 0.12 (3)	1.88 ± 0.04 (2)
0.1	1.03 (1)	-
0.2	1.00 ± 0.08 (4)	1.69 ± 0.15 (3)
1	0.88 ± 0.03 (2)	1.40 ± 0.14 (5)
5	-	1.47 ± 0.14 (5)
25	1.43 (1)	1.57 ± 0.23 (3)

n_H values represent the slopes of log (v/Vm-v) plotted as a function of log (MeGTP) from experiments similar to that shown in figure 2. Values are means of different experiments ± S.D. (number of experiments).

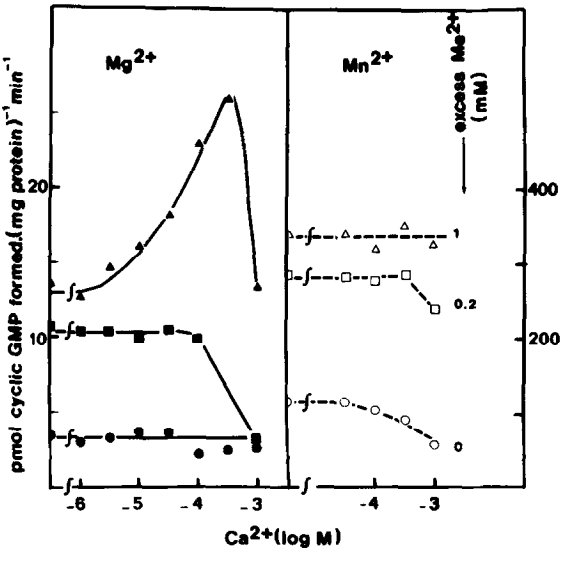

Figure 3. Effect of Ca^{2+} concentration on Mg^{2+}- and Mn^{2+}-dependent guanylate cyclase activities in Triton-treated plasma membranes from rat adipocytes.

Either 3.2 µg or 1.8µg membrane protein were incubated for 30 min. with 0.1 mM MeGTP, in the presence of various excess of Mg^{2+} or Mn^{2+}, respectively.

Our findings can not be accounted for, solely, by the different affinity constants of the metal-nucleotide complexes (16). Free Me^{2+} and GTP and MeGTP complex concentrations have been estimated approximately, assuming that only GTP may bind Me^{2+} species; when excess $MeCl_2$ is as low as 0.2 mM, more than 93% of GTP is present in the complexed form, irrespective of its initial concentration. In reference to the possibility of inhibition by free GTP, the variations in the concentration of this species relative to those of MeGTP appear unable to explain our experimental data.

Therefore, in the absence of substrate hydrolysis (see "Methods"), the activity profiles as a function of MeGTP (fig.2) discriminate two different kinetic behaviours. Thus, at optimal Mn^{2+} concentration, non-purified guanylate cyclase from detergent-treated plasma membranes can exhibit an hyperbolic profile, that is, typical for soluble guanylate cyclase activity (10, for a review). Michaelian kinetics for guanylate cyclase activity derived from particulate fractions has been previously observed only with the sea urchin sperm enzyme purified by affinity chromatography in the presence of Mn^{2+}(17). It is noteworthy that, when using 0.2 mM excess Mn^{2+}, increasing the ionic strength by addition of NaCl (150mM) or Na_2SO_4 (25 mM) restored the apparent positive cooperativity of the enzyme (data not shown). Similarly, sulfate anion was able to induce positive cooperativity of kidney plasma membranes adenylate cyclase activity toward its substrate, MgATP (18).

A biphasic effect of the cations was observed in the adipocyte guanylate cyclase system (fig.1) as is the case with the liver enzyme after NaN_3 activation (19. These observations may be explained by the existence of a binding site for free Me^{2+}(20-22). Basal and activated guanylate cyclases have a much higher affinity for MnGTP than for MgGTP (23,24,this report) and for Mn^{2+} as compared to Mg^{2+} (19,20,this report); therefore Mn^{2+} may contribute in a significant way to guanylate cyclase activity *in vivo*, although tissue Mn^{2+} level is very low (25), as outlined by several authors. On an other hand, considering the redox modulation of guanylate cyclase (8,26), some differences between Mn^{2+} and Mg^{2+}-activities may be explained by the specific involvment of Mn^{2+} in NADPH oxidation (27,28). Indeed, several processes at the adipocyte plasma membrane level are dependent upon the NADH-linked redox state of the membrane, among which the hormone-sensitive adenylate cyclase activity (29,30).

Finally, we have observed activation of plasma membrane guanylate cyclase by low concentrations of calcium, in the presence of Mg^{2+} (fig.3). The enzyme from fibroblasts membranes has a similar behaviour (31). In other conditions, Ca^{2+} was also able to stimulate the particulate guanylate cyclase activity from sea urchin sperm (16). The stimulation by Ca^{2+} further distinguishes Mn^{2+}- and Mg^{2+}-dependent guanylate cyclase activities and suggests a possible role of the latter activity in the Ca^{2+}-dependent intracellular accumulation of cyclic GMP (11,32).

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